World J Microbiol Biotechnol (2006) 22:1295–1299
DOI 10.1007/s11274-006-9175-8

ORIGINAL PAPER

Natural cashew apple juice as fermentation medium for biosurfactant production by *Acinetobacter calcoaceticus*

Maria V. P. Rocha · Adriano H. S. Oliveira · Maria C. M. Souza · Luciana R. B. Gonçalves

Received: 18 January 2006 / Accepted: 29 March 2006 / Published online: 3 May 2006

Abstract The success of biosurfactant production depends on the development of cheaper processes based on the use of low cost raw materials, which account for 10–30% of the overall process cost. In Brazil, the cashew apple agroindustry plays an outstanding role in the local economy. However, only a small part of the pseudofruit produced is used industrially and the amount wasted (about 94%) presents high potential as fermentation media, since it is rich in carbohydrate, fibers, vitamins and minerals salts. In this work, the performance of cashew apple juice (CAJ) as a complex medium for *Acinetobacter calcoaceticus* growth and production of biosurfactant was investigated. The microorganism was able to grow and to produce biosurfactant on a defined culture medium and on CAJ, reducing the surface tension of both media. The biosurfactant also achieved a maximum emulsion index of 80% for kerosene, when defined medium was used.

Keywords Biosurfactants · Cashew apple juice · Submerged fermentation · Agro industrial residues · *A. calcoaceticus*

Introduction

Biosurfactants are surface-active molecules produced by microorganisms that find applications in an extremely wide variety of industrial process involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Lin et al. 1998; Nitschke et al. 2004). Almost all surfactants in use are chemically derived from petroleum, however, naturally surface-active compounds, called biosurfactants, are attracting attention due to several advantages over the chemical surfactants, such as lower toxicity, good biodegradability and ecological acceptability, selectivity and specificity at extreme temperatures, pH and salinity (Nitschke et al. 2004; Ilori et al. 2005).

Nowadays, biosurfactants are not widely used due to high production costs associated with use of expensive substrates and inefficient product recovery methods (Fox and Bala 2000). Therefore, a possible strategy to reduce costs is the use of inexpensive substrates such as agroindustrial wastes, if those residues generally contain high levels of carbohydrates or lipids to support growth and biosurfactant biosynthesis (Nitschke et al. 2005). Natural cashew apple juice (CAJ) is an example of an inexpensive substrate, since it is a by-product of the cashew nut industry.

In the north coast of Brazil, especially in the state of Ceará, the cashew agroindustry has an outstanding role in the local economy. The cashew apple, a pseudofruit or peduncle, is the part of the tree that connects it to the cashew nut, the real fruit and a well known product around the world. The cashew apple is a hard, pear-shaped, small and non climacteric fruit, and is found in three colours: yellow, orange and red. The most commonly commercialized ones are the yellow and red fruits. The edible portion, representing 90% of the fruit, is a pseudofruit rich in vitamin C, flavour and aroma. Internal and external market consumption of cashew nut, in the year of 2004, was about 232,000

M. V. P. Rocha (✉) · A. H. S. Oliveira · M. C. M. Souza · L. R. B. Gonçalves
Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici, Bloco 709, CEP: 60455-760 Fortaleza, CE, Brazil
e-mail: lrg@ufc.br

tonnes. However, only 12% of the total peduncle is consumed ''in natura'' or processed industrially to produce a wide range of products from concentrated juice to desserts. The industrially processed products are basically consumed by the local market and they do not play an important role in the state economy. Furthermore, the majority of cashew apples rot in the soil (Morton and Dowling 1987; Campos et al. 2002; Assunção & Mercadante 2003; Azevedo & Rodrigues 2000). Those facts, together with its rich composition (see Table 1), turn cashew apple juice into an interesting and inexpensive (R$1.00/kg) substrate for several potential applications. Ezeronye (2004) has studied its use as an alternative substrate for wine production and the author concluded that cashew juice was a good choice for commercial scale fermentation. Osho (1995) observed the ability of cashew juice to support the growth of yeasts and suggested it as a feedstock for single cell protein (SCP) and wine production. Another author (Akinwale 2000) studied the use of cashew apple juice to fortify the nutritional quality of some tropical fruits, since it contains a high amount of Vitamin C. In this work, cashew apple juice was investigated as culture medium for the production of biosurfactant using *Acinetobacter calcoaceticus*. Selection and utilization of CAJ for this purpose would offer an alternative means of use, especially during peak harvest periods.

The biosurfactant produced by *A. calcoaceticus*, emulsan, is an extra cellular polyanionic amphipathic heteropolysaccharide. Its amphipathic and polymeric characteristics provide an emulsifying as well as a stabilizing activity for oil/water systems (Shabtai 1990). Due to its native properties as dispersant and its biodegradability, it is widely used in many industrial fields, for instance, for cleaning oil-contaminated vessels and oil-spill management, microbial enhanced oil recovery, as wells as in creams, lotions, soaps and shampoo, as well as in the food and agricultural industries (Choi et al. 1996; Patil and Chopade 2001). Emulsan has been studied in detail (Pail and Chopade 2001; Panilaitis et al. 2002; Bach et al. 2003; Shabtai 1990; Rosenberg and Ron 1997; Choi et al. 1996), but there is no report of biosurfactant production using agroindustrial residues as alternative substrates.

Therefore, the aim of this work was to analyse the biosurfactant production by *Acinetobacter calcoaceticus*, and other related parameters, in a defined mineral and a complex medium, cashew apple juice, and compare the results obtained in order to determine the potential of this agroindustrial waste to replace synthetic medium.

Table 1 Cashew apple juice composition

Parameter	Content
Vitamin C $\times 10^3$ (mg/l)[a,b]	13.53–37.27
Brix[b]	7.4
PH[a,b]	3.8–4.2
Malic acid (g/l)[b]	0.04
Total tannins $\times 10^3$ (g/l)[b]	0.06
Condensed tannins $\times 10^3$ (g/l)[b]	0.02
Calcium $\times 10^3$ (g/l)[a]	0.09–0.54
Phosphorus $\times 10^3$ (g/l)[a]	0.61–2.14
Iron $\times 10^3$ (g/l)[a]	0.02–0.07
Carotene $\times 10^6$ (g/l)[a]	3.0–75.51
Carbohydrates (g/l)[a]	0.9–0.97
Reducing sugars (%)[c]	10.7
Non reducing sugars (%)[c]	0.4
Starch (%)[c]	8.5–2.7
Alanine (mM)[d]	3.36
Serine (mM)[d]	2.73
Phenylalanine (mM)[d]	1.76
Leucine (mM)[d]	1.78
Glutamic acid (mM)[d]	1.48
Aspartic acid (mM)[d]	0.88
Proline (mM)[d]	1.59
Tyrosine (mM)[d]	1.15

[a]Morton and Dowling (1987)

[b]Campos et al. (2002)

[c]Souza et al. (2002)

[d]Oliveira et al. (2002)

Materials and methods

Microorganisms

Acinetobacter calcoaceticus RAG-1, the microorganism used in this work, was kindly donated by Dr. Flávio Tavares from the Genetic Department of Escola Superior de Agricultura Luiz de Queiroz (ESALQ/USP). *A. calcoaceticus* was maintained on YEPD agar (2% of glucose 1% de peptone and 1% yeast extract) at 4 °C.

Culture Medium and culture conditions

In this work, two culture media were investigated for biosurfactant production: a defined mineral medium, containing KH_2PO_4 1.834% (w/v); K_2HPO_4 0.6% (w/v); $MgSO_4{\cdot}7H_2O$ 0.02% (w/v); $(NH_4)_2SO_4$ 0.4% (w/v), and natural cashew apple juice (CAJ), see Table 1 for average composition.

The CAJ was withdrawn by compressing the cashew apple (*Anarcardium occidentale* L.). Afterwards, the pH was adjusted to 7.0 and the medium was exposed to ultraviolet radiation for 1 h for sterilization in order to avoid loss of heat-labile components. The mineral medium was sterilized at 121 °C for 15 min.

Inocula were prepared on a YEPD agar slant incubated for 24 h at 30 °C. The culture was inoculated into 500-ml Erlenmeyer flasks containing 50 ml of defined medium or CAJ.

The flasks were incubated on a rotary shaker (Tecnal – TE240, BR) at 30 °C 150 rev/min for 5 days. At defined intervals, fermentation parameters, such as biomass concentration, surface tension, total reducing sugar and pH, were monitored.

Analytical methods

Biomass

Cell growth was determined by measuring the optical density of samples, using a UV-visible spectrophotometer (20 Genesis, BR) at 540 nm.

Substrate Consuming

Total reducing sugars were determined colorimetrically by dinitrosalicylic acid (DNS) method (Lima Lobato et al. 2002). Usually 1.4 ml of DNS was added to 1.4 ml of sample. The mixture was heated in a water bath at 100 °C for 5 min and potassium sodium tartrate was subsequently added. Afterwards, the solution was cooled to room temperature and the absorbance was measured at 540 nm. An appropriate calibration curve was used to convert absorbance to concentration.

Emulsification activity

Emulsifying activity was performed according to Cooper and Goldenberg (1987) with slight modifications: 2 ml of cell free supernatant was added to 2 ml of kerosene, containing 0.2 ml of pink dye and the mixture was vortexed for 2 min. After 24 h, the height of emulsion layer was measured. The emulsifying activity (E_{24}) was calculated using equation 1 (Wei et al. 2005).

$$E_{24}(\%) = \frac{H_{EL}}{H_S} * 100 \quad (1)$$

Where H_{EL} is the height of the emulsion layer and H_S is the height of total solution.

Surface tension measurement

Surface tension was determined with Tensiometer (Torsion Balance of White Electrical Instrument) at 30 °C when biosurfactant was produced on a mineral medium. The surface tension determinations were replicated and performed using cell free supernatants obtained after centrifugation.

When CAJ medium was used, surface tension was determined in relation to water using stalagmometer. The surface tension (σ) was calculated using equation 2 (Adamczak and Bednarski 2000).

$$\sigma = \frac{n_{water}}{n} * \sigma_{water} \quad (2)$$

Where σ_{water} is the surface tension of water at 20 °C; n and n_{water} is the amount of droplets of the sample and water, respectively.

Results and discussion

Biosurfactant production with A. calcoaceticus in mineral media

Biosurfactant production by *Acinetobacter calcoaceticus* was carried out in batch assays and process parameters were monitored. Figure 1 shows microbial growth, biosurfactant production and emulsifying activity (E_{24}) of the fermentation broth using a defined medium and *Acinetobacter calcoaceticus*.

The highest biomass yield was obtained after 24 h of incubation, which remained in stationary phase up to 96 h. At this phase, the surface tension of the broth was reduced by 11% just after biomass addition to the culture medium, which continued unchanged until 96 h. Some authors (Lima Lobato et al. 2002; Ferraz et al. 2002) report the same behaviour and explain that even in the presence of a small concentration of biosurfactant, the critical micellar concentration may be achieved, above which no further variation in the surface tension can be observed. Moreover, some microbial cells present high superficial hydrophobicity, for instance *Acinetobacter calcoaceticus* (Nitschke and Pastore 2002), which would explain the rapid decrease in surface tension at the beginning of the assay.

It was observed (Fig. 1) that no emulsifying activity was detected before 50 h of cultivation and after 90 h, a E_{24} value of 85.7% was obtained, showing that the supernantant was able to emulsify kerosene. It can be also observed that the emulsifying activity of the supernatant coincides with the microorganism stationary phase, what indicates that the biosurfactant is an secondary metabolite.

Biosurfactant production with A. calcoaceticus in cashew apple juice (CAJ)

Figure 2 show the time course behaviour of cell growth, substrate uptake (total reducing sugars), surface tension and emulsifying activity (E_{24}) of supernatant

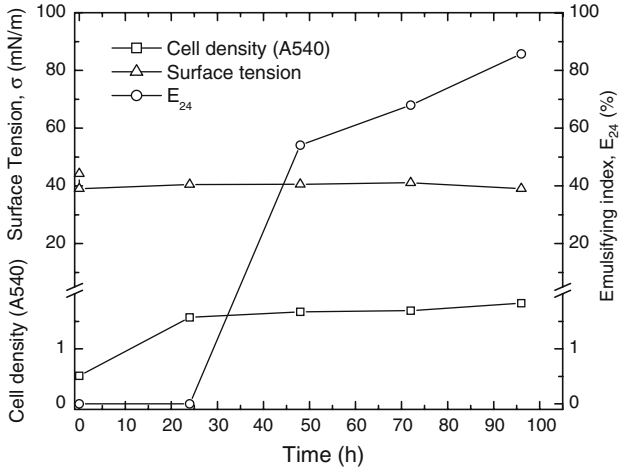

Fig. 1 Time course of biosurfactant production by *Acinetobacter calcoaceticus* in a defined mineral media at 30°C and pH 7.0: ((□) Cell growth ($\ln\left(D/D_0\right)$) (○) emulsifying activity (%) and (Δ) Surface Tension (mN/m)

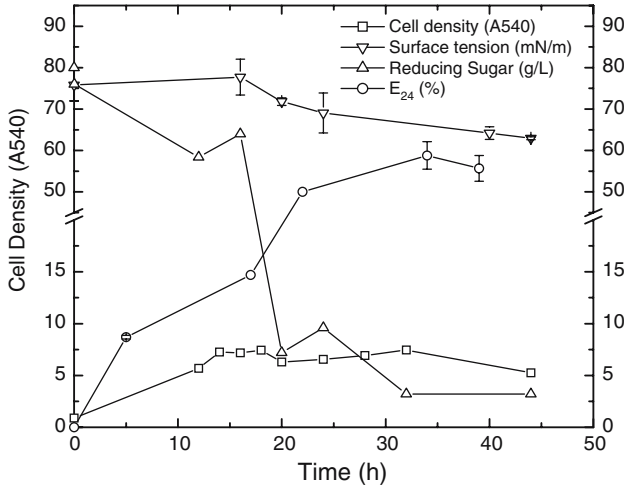

Fig. 2 Time course of cell growth, surface tension of supernatant and substrate uptake, total reducing sugars) during *Acinetobacter calcoaceticus* cultivation in a complex medium (cashew apple juice) medium at 30 °C and pH 7.0

during the cultivation of *Acinetobacter calcoaceticus* in natural cashew apple juice (CAJ). It can be observed that cell growth is a typical cell growth curve. Moreover, substrate uptake was concentrated on the log phase and the stationary phase was achieved when substrate was exhausted. The same behaviour was observed for several authors studying different microorganisms (Nitschke and Pastore 2002; Lima Lobato et al. 2002; Santa Anna et al. 2001). A comparison between cell growth, surface tension and substrate uptake showed that the biosurfactant was produced after 15 h of cultivation, which coincides with the exhaustion of substrate and the beginning of the stationary phase. Several biosurfactants were recognized as secondary metabolites, while others were considered growth-associated (Desai and Banat 1997). In this study, the observed behaviour is typical of secondary metabolites.

It can be also observed (Fig. 2) that an emulsifying activity value of 58.8% was obtained, after 34 h, showing that the supernantant was able to emulsify kerosene.

Conclusions

In this study, cashew apple juice (CAJ), an agroindustrial residue, was investigated for the production of biosurfactant by fermentation using *Acinetobacter calcoaceticus.* The total reducing sugars consumption of CAJ implies that it is a viable substrate for biosurfactant production by *A. calcoacetucis*. This initial study indicates that traditional carbon sources for biosurfactant production may be replaced by CAJ. Moreover, the use of cashew apple juice as a culture medium would provide an alternative of waste management for the productive chain of cashew nut, an important industrial segment in Northeast Brazil.

Acknowledgements The authors would like to thank FINEP, CNPq, ANP (from Brazil), for the financial support that made this work possible. Dr. Flávio Tavares (ESALQ/USP) is gratefully recognized by donation of microorganism.

References

Adamczak M, Bednarski W (2000) Influence of medium composition and aeration on the synthesis of biosurfactants produced by *Candida Antarctica*. Biotechnol Lett 22:313–316

Akinwale TO (2000) Cashew apple juice: its use in fortifying the nutritional quality of some tropical fruits. Eur Food Res Technol 211:205–207

Assunção RB, Mercadante AZ (2003) Carotenoids and ascorbic acid composition from commercial products of cashew apple (*Anacardium occidentale* L.). J Food Composition Anal 16:647–657

Azevedo DCS, Rodrigues A (2000) SMB chromatography applied to the separation/purification of fructose from cashew apple juice. Brazilian J Chem Eng 17:507–516

Bach H. Berdichevsky Y, Gutnick D (2003) An exocellular protein from the oil-degrading microbe *Acinetobacter venetianus* RAG-1 enhances the emulsifying activity of the polymeric bioemulsifier emulsan. Appl Environ Microbiol 69:2608–2614

Campos DCP, Santos AS, Wolkoff DB, Matta VM, Cabral LMC, Couri S (2002) Cashew apple juice stabilization by microfiltration. Desalination 148:61–65

Choi JW, Choi HG, Lee WH (1996) Effects of ethanol and phosphate on emulsan production by *Acinetobacter* RAG-1. J Biotechnol 45:217–225

Cooper DG, Goldenberg BG (1987) Surface-active agents from 2 Bacillus species. Appl Environ Microbiol 53:224–229

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64

Ezeronye OU (2004) Nutrient utilization profile of *Saccharomyces cerevisiae* from palmwine in tropical fruit fermentation. Antonie Van Leeuwenhoek 86:235–240

Ferraz C, De Araujo AA, Pastore GM (2002) The influence of vegetable oils on biosurfactant production by *Serratia marcescens*. Appl Biochem Biotechnol 98:841–847

Fox SL, Bala GA, (2000) Production of surfactant from Bacillus subtilis ATCC 21332 using potato substrate. Bioresour Technol 75:235–240

Ilori MO, Amobi CJ, Odocha AC (2005) Factor affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. Chemosphere 61:985–992

Lima Lobato AKC, Macedo GR, Magalhães MMA, Bezerra MS (2002) Estudo cinético da produção de biossurfactante. In Proceedings of XIV Congresso Brasileira de Engenharia Química (In Portuguese)

Lin SC, Lin KG, Lo CC, Lin YM (1998) Enhanced biosurfactant production by a Bacillus licheniformis mutant. Enzyme Microbial Technol 23:267–273

Morton JF, Dowling CF (1987) Fruits of warm climates, Creative Resources Systems, pp 239–240, ISBN: 0961018410

Nitschke M, Costa SGVAO, Hadad R, Gonçalves LA, Eberlin MN, Contiero J (2005) Oil wastes as unconventional substrates for rhamnolipd biosurfactant production by *Pseudomonas aeoginosa* LBI. Biotechnol Progr 21:1562–1566

Nitschke M Ferraz C, Pastore GM (2004) Selection of microorganisms for biosurfactant production using agroindustrial wastes. Brazilian J Microbiol 35:81–85

Nitschke M, Pastore GM, (2002) Biossurfactantes: Propriedades e aplicações. Química Nova 25:772–776

Oliveira MEB, Oliveira GSF, Maia GA, Moreira RA, Monteiro ACO (2002) Aminoácidos livres majoritários no suco de caju: variação ao longo da safra. Revista Brasileira de Fruticultura 24:133–137

Osho A (1995) Evaluation of cashew apple juice for single cell proteins and wine production. Nahrung-Food 39:521–529

Panilaitis B, Johri A, Blank W, Kaplan D, Fuhrman J, (2002) Adjuvant activity of emulsan, a secreted lipolysaccharide from *Acinetobacter calcoaceticus*. Clin Diagnostic Lab Immunol 9:1240–1247

Patil JR, Chopade BA (2001) Studies on biomulsifier production by *Acinetobacter* strains isolated from healthy human skin. J Appl Microbiol 91:290–298

Rosenberg E, Ron EZ (1997) Bioemulsans: microbial polymeric emulsifiers. Curr Opin Biotechnol 8:313–316

Santa Anna LM, Sebastian GV, Pereira N Jr., Alves TLM, Menezes EP, Freire DMG (2001) Production of biosurfactant from a new promisins strain of *Pseudomonas aeruginosa* PA1. Appl Biochem Biotechnol 91–93:459–467

Shabtai Y (1990) Production of exopolysaccharides by *Acinetobacter* strains in a controlled fed-batch fermentation process using soap stock oil (SSO) as carbon source. Int J Biol Macromol 12:145–152

Souza PA, Simões AN, Menezes JB, Andrade JC, Freitas DF, Mendonça FVS (2002) Caracterização Química de Pedúnculos de Caju 'CCP-76' em Diferentes Estádios de Desenvolvimento. Proceedings of the XVII Congresso Brasileiro de Fruticultura (In Portuguese)

Wei YH, Chou CL, Chang JS (2005) Rhamnolipid production by indigenous *Pseudomonas aeroginosa* J4 originated from petrochemical wastewater. Biochem Eng J 27:146–154